CHAPTER 6

Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: different poly(3-hydroxyalkanoates) are stored in separate granules.

Hans Preusting, Jaap Kingma, Gjalt Huisman, Alexander Steinbüchel[#], and Bernard Witholt

Department of Biochemistry, University of Groningen
Nijenborgh 4, 9747 AG Groningen, The Netherlands.
[#] Institut für Mikrobiologie der Georg-August-Universität Göttingen,
Grisebachstraße 8, D-3400 Göttingen, Republic of Germany.

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SUMMARY

When *Pseudomonas oleovorans* (GPo1) is grown on sodium octanoate under ammonium limiting conditions, it is able to accumulate a copolyester consisting of medium chain length 3-hydroxyalkanoic acids (PHA_m). 3-Hydroxybutyrate is only incorporated in trace amounts. When *P. oleovorans* is equipped with the PHB biosynthetic genes of *Alcaligenes eutrophus* (GPo1[pVK101::PP1]), it forms a polyester containing major amounts of 3-hydroxybutyrate. The resulting polymer however is a blend of PHA_m and PHB, rather than a copolymer of 3-hydroxybutyrate and medium chain length 3-hydroxyalkanoic acids [11].

To establish whether PHA_m and PHB molecules are stored in the same or separate granules by this recombinant *P. oleovorans* strain, we studied polymer forming cells by freeze-fracture electron microscopy. This approach is possible because previous freeze-fracture electron microscopy studies on PHA_m and PHB accumulating strains have shown that PHA_m and PHB granules can be distinguished from each other: PHA_m granules form mushroom-like structures, whereas PHB granules form needle structures during freeze-fracturing.

In this paper we show that stationary phase cells of GPo1[pVK101::PP1] contained both mushroom and needle-like structures, indicating that PHA_m and PHB chains were stored in separate granules. To be able to determine whether the separation of PHA_m and PHB is complete, the respective granules were separated on sucrose gradients. A total cell extract of GPo1[pVK101::PP1], which was subjected to sucrose gradient centrifugation, revealed two white bands of different densities: the upper band with a density of 1.05 g/mL consisted exclusively of PHA_m granules while the lower band with a density of 1.19 g/mL consisted of PHB granules only. Thus, when bacteria synthesize both PHA_m and PHB, the resulting polymer chains are segregated completely and stored in separate granules.

INTRODUCTION

Poly(3-hydroxyalkanoates) (PHAs) is a family name for polyesters of prokaryotic origin, which serve as storage material and are accumulated by a wide range of microorganisms. The largest part of this set of microorganisms accumulates poly(3-hydroxybutyrate) (PHB) or heteropolymers of other short chain 3-hydroxy acids in addition to 3-hydroxybutyrate (PHA_s) [1,2]. In the early 1980's, nearly 60 years after the discovery of PHB by Lemoigne [3], de Smet *et al.* [4] found that a second class of PHAs is accumulated by *Pseudomonas*

oleovorans, which forms a polyester containing primarily 3-hydroxyoctanoate monomers, after growth on *n*-octane. It was subsequently found that the PHAs formed by *P. oleovorans* after growth on C_6 to C_{12} *n*-alkanes or corresponding fatty acids consist of medium chain length (C_6-C_{12}) 3-hydroxy acids (PHA_m) of which the composition is determined by the length of the carbon source used [5,6]. Recently, it has been shown that the formation of PHA_m is restricted to the rRNA I homology group fluorescent pseudomonads [7,8].

Despite the large variety of microorganisms capable of accumulating intracellular storage materials in the form of polyesters, PHA_s-producers have never been reported to incorporate significant amounts of medium chain length 3-hydroxy acids into their polymer. Conversely, PHA_m-accumulating strains only incorporate trace amounts of short chain 3-hydroxy acids in their PHA [9,10]. These results are presumably due to the specificity of one or more of the enzymes involved in PHA_s and PHA_m synthesis.

However, when a PHA_m-producing strain is equipped with the PHB biosynthetic genes, it is able to accumulate high levels of 3-hydroxybutyrate. It was demonstrated that these short chain monomers are not incorporated into the PHA_m polymer, but into a separate polymer, namely PHB. As a result, this recombinant *P. oleovorans* strain forms blends instead of copolymers [11]. Since these polyesters are stored in granules, such a blend might be formed *in vivo*, with PHB and PHA_m chains stored in one single granule or the blend might be formed during the isolation procedure from PHB and PHA_m chains which were originally stored in separate granules.

Freeze-fracture electron microscopy experiments on whole cells and isolated granules revealed that PHB and PHA_m granules could be stretched following the fracture procedure. After stretching, the PHB granules in *B. cereus* are needle-shaped [12], while the PHA_m granules of *P. oleovorans* are mushroom-shaped [4,13]. This suggests that freeze-fracture electron microscopy can be used to discriminate between a PHB or PHA_m granule *in vivo*.

In this paper we describe the morphology of PHA_m and PHB granules after freeze-fracturing of a *P. oleovorans* recombinant, which is equipped with the PHB biosynthetic genes of *A. eutrophus*. These experiments show that PHA_m and PHB chains are stored in separate granules. We corroborate this finding by showing that PHA_m and PHB granules can be separated by density gradient centrifugation of cell free extracts of the recombinant *P. oleovorans*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Pseudomonas* and *Escherichia coli* strains as well as the plasmids used in this study are listed in Table I.

Table I. Bacterial strains and plasmids used in this study.

strains/plasmids	relevant genotype or phenotype	source/ref.
Strains		
P. oleovorans		
GPo1	PHA^+, PHB^-	[14]
GPo1[pVK101::PP1]	PHA^+, PHB^+, Km^r	[11]
P. putida		
GPp104	PHA^-, PHB^-	[15]
GPp104[pVK101::PP1]	PHA^-, PHB^+, Km^r	this study
Plasmids		
pRK2013	Km^r, Tra^+, ColE1 replicon	[16]
pVK101::PP1	Km^r, PHB-biosynthetic genes	[17]

Conjugations. Mating of the recipient strain with the *E. coli* donor strain was carried out using the helper plasmid pRK2013 [16]. The transconjugants were isolated on minimal medium plates containing 50 μg/mL kanamycin (Km).

Growth conditions. The recombinant *Pseudomonas* strains were precultured on LB-medium supplemented with 50 μg/mL Km at 30°C for about 8 hours. 1 mL of the resulting suspension was transferred to 1 L Erlenmeyer flasks containing 200 mL 1/10NE2 medium [15] supplemented with 1mM $MgSO_4.7H_2O$, 0.1 % (v/v) MT microelements [5], 50 μg/mL Km and 20 mM sodium octanoate, which serves as sole carbon and energy source. The culture was incubated at 30°C and 200 rpm for about 50-60 hours. After 8 hours samples were taken at regular intervals for determination of the PHA composition and for freeze-fracture electron microscopy.

The wild-type *P. oleovorans* GPo1 was also cultured as descibed above omitting the antibiotic selection marker.

Biomass determination. Cell densities, expressed as grams of cell dry weight per liter of water phase, were calculated as described before [18].

PHA analysis. The monomeric composition of the accumulated polyesters was determined as described before [5].

Freeze-fracture electron microscopy. Freeze-fracture electron microscopy was carried out as described before [4].

Density gradient centrifugation. Cells were cultured using similar conditions as above, harvested (10 min. 7000 g, 4°C) and resuspended in 10 mM Tris.HCl (pH 8.0) to a density of about 20 mg/mL. The cells were converted to spheroplasts by a modification of the procedure described by Witholt *et al.* [19]. To this end, the cell suspension was diluted twofold with 0.5 M sucrose (pH 8.0) (t=0 min.). After 1 minute egg-white lysozyme was added to a final concentration of 90 μg/mL. At t=2 min. EDTA was added to a concentration of 1 mM and at t=8 min. the spheroplasts were stabilized by the addition of Mg^{++} (final concentration of 20 mM). The spheroplasts were harvested (5 min., 2750 g, 4°C) and resuspended in 10 mM Tris.HCl (pH 8.0) which was supplemented with 0.1 mg/mL RNase and DNase. To release the intracellular polymer, the spheroplasts were lysed in the French press.

For determination of the density of polymer granules, 1.1 mL of total cell lysate was layered on a sucrose gradient of 15 to 55 % (8 layers of 1.3 mL and 0.5 mL of the bottom (55 %) layer). The gradients were spun for 68 hours at 110,000 g in a SW 41 rotor (Beckman, U.S.A.), after which they were fractionated (0.5 mL per fraction). The refractive index and optical density at 450 nm of each fraction were measured and the polymer containing fractions (white bands which showed a relatively high OD 450) were pooled. The granule suspension was diluted threefold with 10 mM Tris.HCl (pH 8.0) and centrifuged (Eppendorf, 10 min., 4°C). The granule pellet was resuspended in 10mM Tris.HCl (pH 8.0) and after lyophilization analyzed for PHA.

The relative density of whole bacterial cells was measured using Percoll (Sigma, U.S.A.) gradients as described by Guerrero *et al.* [20].

RESULTS

Growth and morphology of PHA_m and PHB producing strains

Pseudomonas oleovorans GPo1 and *Pseudomonas putida* GPp104[pVK101::PP1] were grown on sodium octanoate under ammonium limiting conditions. Figure 1 shows the growth and polyester accumulation of these strains. As expected, *P. oleovorans* GPo1 accumulated PHA_m, a polyester consisting of medium chain length 3-hydroxyalkanoates of which the ratio did not change during the fermentation: 92.5 % (w/w) 3-hydroxyoctanoate and 7.5 % (w/w) 3-hydroxyhexanoate. After about 15 hours of cultivation, the PHA_m content of the cells had already reached its maximum value of about 50 % of the cell dry weight and remained

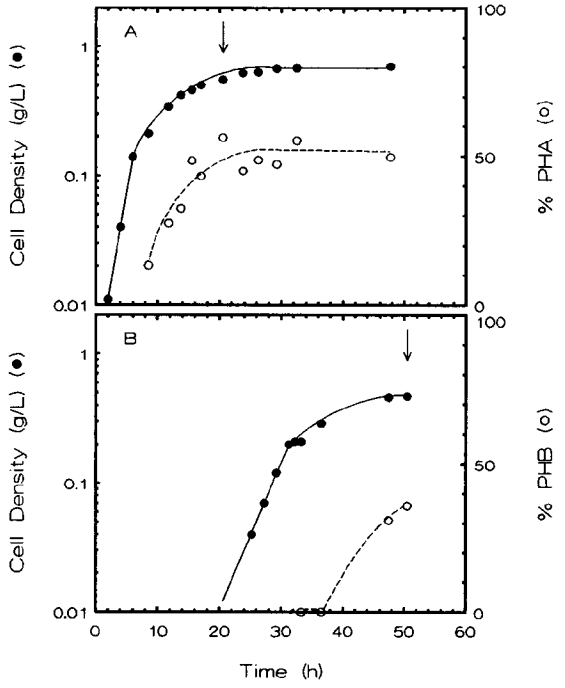
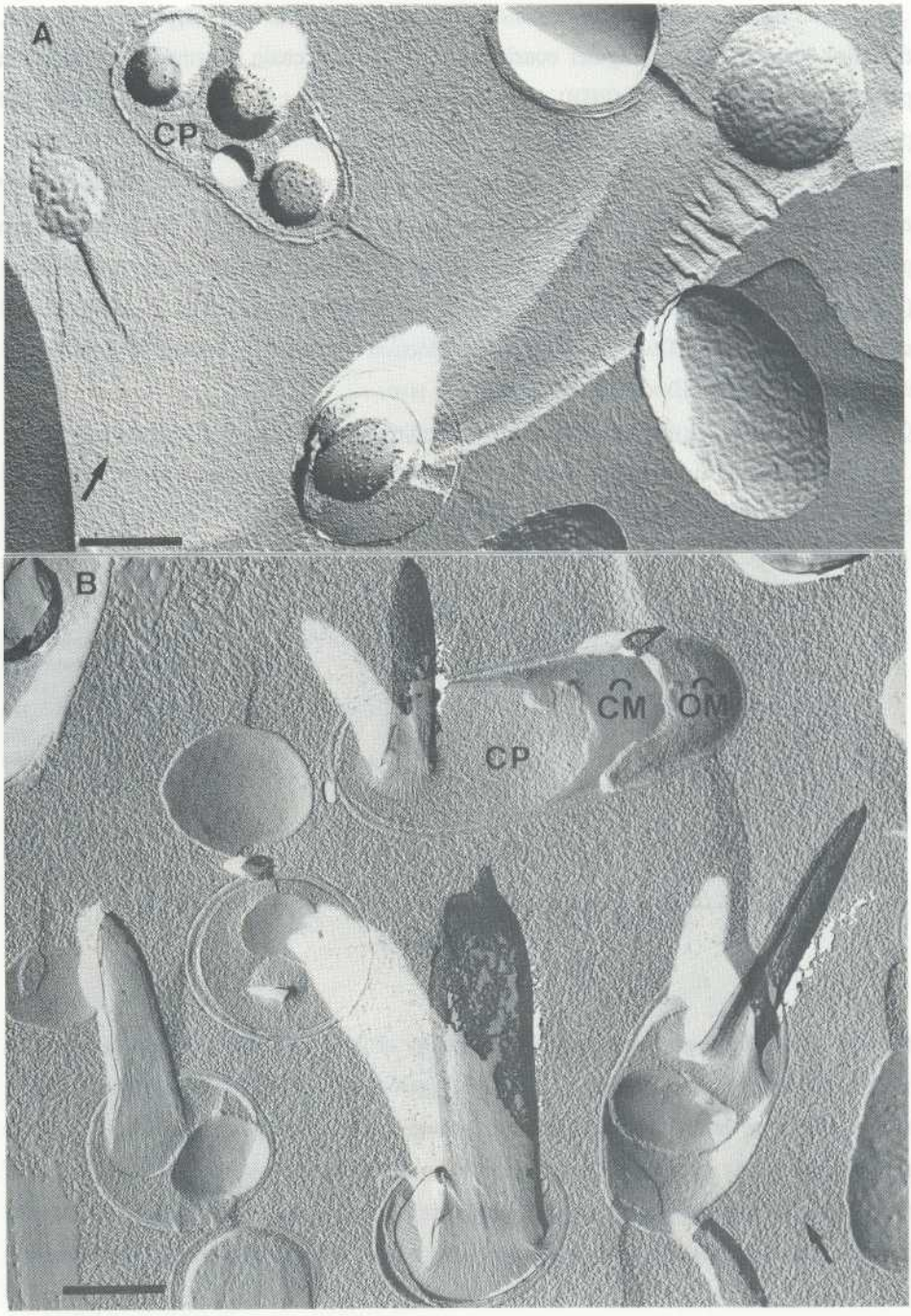


Figure 1. *Growth and poly(3-hydroxyalkanoate) formation by* P. oleovorans *GPo1 and* P. putida *GPp104[pVK101::PP1]. The arrow indicates the time of freeze-fracture electron microscopy. (A) GPo1, % PHA is expressed as g PHA_m per 100 g cell dry weight. (B) GPp104[pVK101::PP1], % PHB is expressed as g PHB per 100 g cell dry weight.*

Figure 2. *Freeze-fracture electron micrograph of* P. oleovorans *GPo1 and* P. putida *GPp104[pVK101::PP1] cells in the stationary phase after growth on sodium octanoate. CP = cytoplasm; CM = the convex cytoplasmic membrane fracture face; OM = the convex outer membrane fracture face; the arrow indicates the direction of shadowing; the bar represents 0.5 μm.*
(A) GPo1 cells containing PHA_m granules which show a mushroom-type deformation.
(B) GPp104[pVK101::PP1] cells containing PHB granules which show needle-type deformation.


A
CP
B
CM
OM
CP

constant during the rest of the fermentation (Fig. 1A). *P. putida* GPp104[pVK101::PP1] on the other hand formed a polymer consisting of only short chain 3-hydroxyalkanoates, in particular 3-hydroxybutyrate (PHB). This strain showed a long lag time before growth and polymer formation started (Fig. 1B).

Samples taken at the points indicated by arrows in Fig. 1 were examined by freeze-fracture electron microscopy. When a polymer granule was stretched as a result of the freeze-fracture procedure, the PHA_m granules in *P. oleovorans* appeared as mushroom-shaped deformations (Fig. 2A), while PHB granules in GPp104[pVK101::PP1] were deformed to needle-shaped structures (Fig. 2B). Thus, freeze-fracture electron microscopy makes it possible to distinguish between PHB and PHA_m granules *in vivo*.

Growth and morphology of a polyester blend forming recombinant of *P. oleovorans*

Pseudomonas oleovorans GPo1[pVK101::PP1], which is able to form blends of PHA_m and PHB [11], was grown under similar conditions as described above.

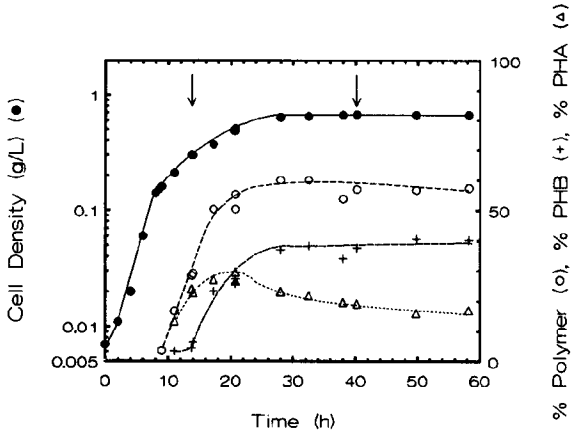


Figure 3. *Growth and polyester formation of* P. oleovorans *GPo1 [pVK101::PP1]. The arrows indicate the times of freeze-fracture electron microscopy. % Polymer (= %PHA_m + %PHB), % PHB and % PHA are expressed as g per 100 g cell dry weight.*

Figure 3 shows the growth and polyester formation of this strain using sodium octanoate as carbon and energy source. GPo1[pVK101::PP1] grew to a final cell density of 0.7 g/L similar to the wild-type host strain without the plasmid. The maximum amount of polymer accumulated by this recombinant strain was similar to that of the host strain, GPo1. It is clear from Fig. 3 that the composition of the polymer changed continuously during the fermentation. Whereas 3-hydroxyoctanoate was the predominant monomer in the beginning

of the accumulation phase, 3-hydroxybutyrate became predominant about 11 hours after the culture had entered the stationary phase.

Freeze-fracture electron microscopy of GPo1[pVK101::PP1] culture samples taken at the times indicated with arrows in Fig. 3 are shown in Fig. 4A and 4B, respectively. Panel A (14 hours of cultivation) shows an example of a cell that contained deformed granules of both the mushroom-type and the needle-type, whereas panel B (40 hours of cultivation) shows cells that contain either deformations of the mushroom-type or needle-type. In some cases, in particular for large deformations, it was difficult to discriminate between the two types of deformations and such granules were counted as undefined. Table II reveals that, after analysis of many such micrographs of cell harvested at both time-points, most fractured cells showed only one type of deformation and that the situation in which a fracture showed both types of deformations was relatively rare. This might indicate that few cells contain both types of granules. It could also be due to the fact that the single fracture plane through a cell, which shows only one or a few granules, cannot show which granule types are located above or below the fracture plane. Thus, most fracture faces will necessary show granules of only one type. This point is further addressed below, via cell density measurements.

Table II. Analysis of the deformations observed in freeze-fracture electron micrographs of Gpo1[pVK101::PP1]. Cells were examined after cultivation for 14 and 40 hours (n = number of cells analyzed per sample).

Time (h)	n	Number of cells containing deformed granules of			
		mushroom type	needle type	mushroom and needle type	undefined type
14	40	23	13	3	1
40	39	7	24	5	3

It is clear from Table II that the ratio between the two types of deformations is consistent with the polymer compositions at the time of freeze-fracturing. At 14 hours of fermentation, when PHA_m was the predominant polymer, the number of mushroom-type deformations was higher than the needle-shaped deformations and at 40 hours of incubation, when the cells contained primarily PHB, needle-shape deformations were dominant.

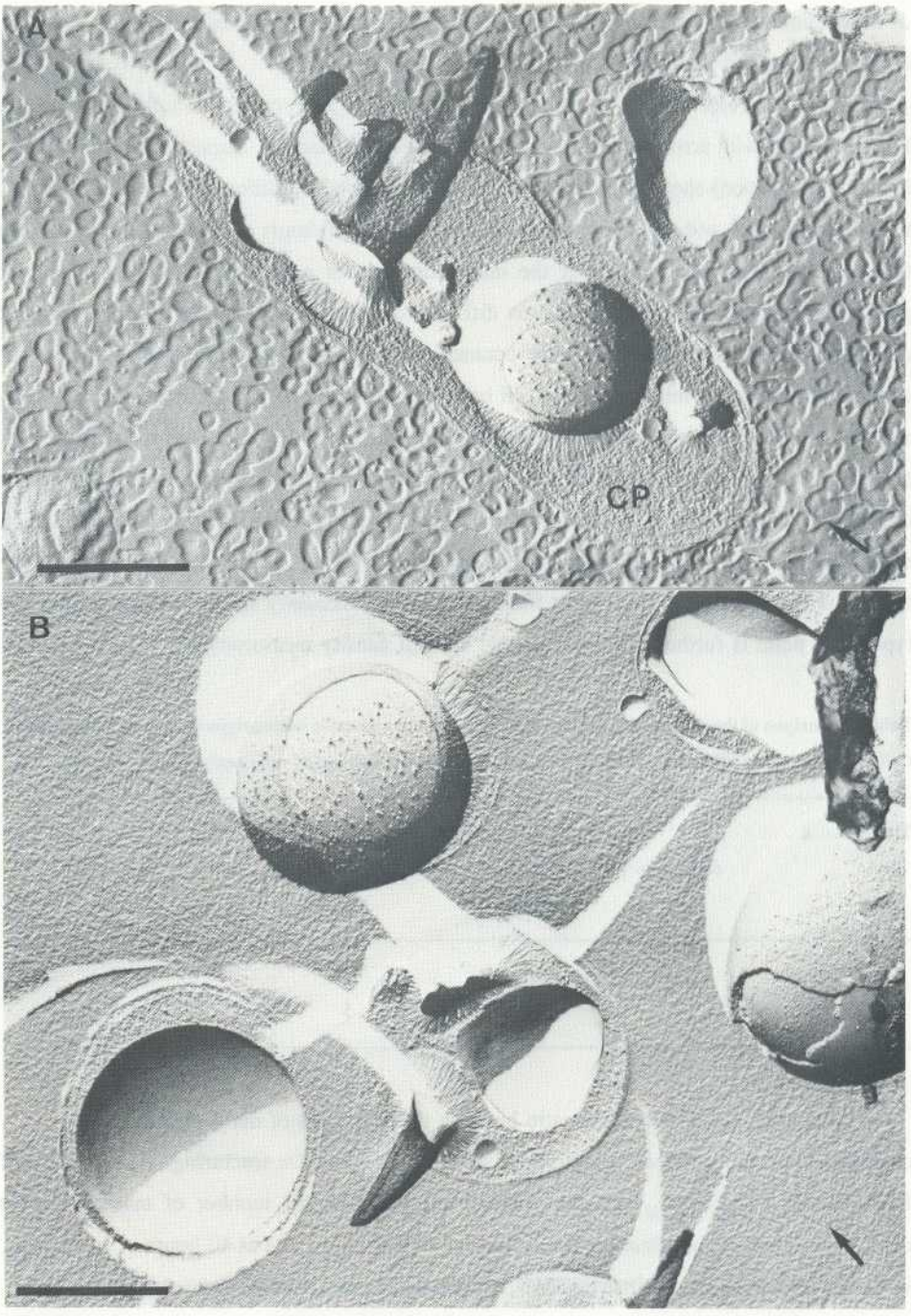

A
CP
B

Figure 4. Freeze-fracture electron micrograph of GPo1[pVK101::PP1] cells after growth on sodium octanoate. CP = cytoplasm; the arrow indicates the direction of shadowing; the bar represents 0.5 μm. (A) Cell after 14 hours of incubation shows both mushroom-type and needle-type deformations. (B) Cells after 40 hours of incubation. One of the cells shows a mushroom-type deformation, whereas the other one shows needle-type deformations. The cell in the lower left corner of the micrograph shows the concave fracture face which remained after removal of a polymer granule.

Thus, freeze-fracture electron microscopy indicated that PHB and PHA_m are stored in separate granules in GPo1[pVK101::PP1] when this strain was grown on sodium octanoate under ammonium limiting conditions. It is not clear from these micrographs however, whether PHA_m and PHB chains are completely separated or whether PHA_m granules contain traces of PHB or vice versa. Therefore, the granules were separated on a sucrose gradient to permit more detailed analysis.

Separation of polyester granules by sucrose density gradient centrifugation

To establish whether PHA_m and PHA_s (=PHB) granules could be separated by sucrose density gradient centrifugation, the density of polymer granules of *P. oleovorans* GPo1 and *P. putida* GPp104[pVK101::PP1] was determined. Cell extracts, containing cell membranes, polymer granules and undisrupted cells, were layered onto a 15-55 % sucrose gradient. After centrifugation, the polymer granules appeared as a white band in the gradient. It was determined that the PHB granules of GPp104[pVK101::PP1] had a density of 1.19 g/mL and the PHA_m granules of GPo1 had a density of 1.05 g/mL. The small peak in OD 450 at a density of 1.20 g/mL in the cell extract of GPo1 was not due to PHB granules as determined by GLC analysis and its identity remains unknown (Fig. 5).

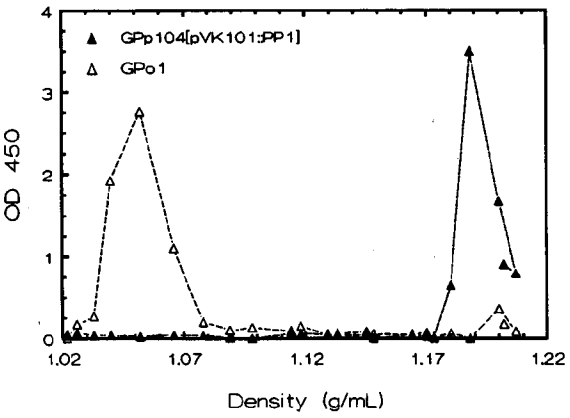


Figure 5. Sucrose density gradient centrifugation of total cell extracts of GPo1 which produces only PHA_m and GPp104[pVK101::PP1] which produces only PHB. The GPo1 extract shows a white band at a mean density of 1.05 g/mL, while the GPp104 [pVK101::PP1] extract shows a band at a mean density of 1.19 g/mL.

The difference in density between PHA_m and PHB granules allowed separation of the different types of granules observed in the recombinant *P. oleovorans* by sucrose density gradient centrifugation.

To this end, GPo1[pVK101::PP1] was grown to a density of 0.5 g/L which resulted in equal amounts of PHA_m and PHB (Fig. 3 at t=20 hours). After cell disruption, the total cell extract was layered onto a 15-55 % sucrose gradient. After centrifugation, two white bands appeared: one in the top of the gradient, which has the same density as the PHA_m granules of GPo1, and one in the bottom of the gradient, which has a density similar to that of the PHB granules of GPp104[pVK101::PP1]. In between the two bands virtually no material had accumulated (Fig. 6).

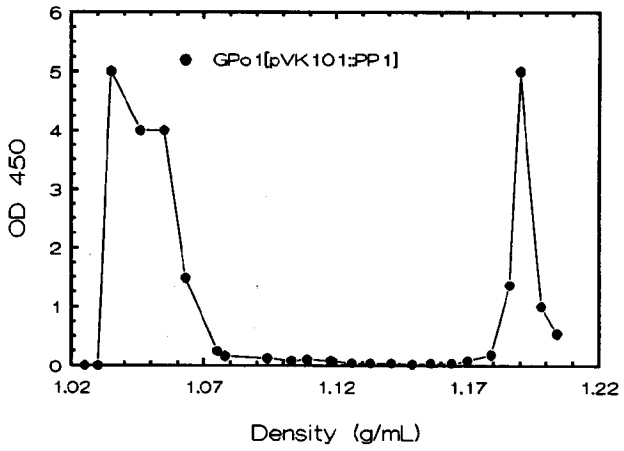


Figure 6. Sucrose density gradient centrifugation of a total extract of GPo1[pVK101::PP1], which produces both PHA_m and PHB. This gradient shows two white bands at mean densities of 1.05 and 1.19 g/mL, respectively.

The white bands were isolated and analyzed for their relative monomer composition. Table III shows that the low density band consisted of 3-hydroxyhexanoate and 3-hydroxyoctanoate in a ratio which was found before for PHA_m [5,6,7,8,9,10]. The high density band consisted of 3-hydroxybutyrate and traces of 3-hydroxyoctanoate, of which the origin is unknown.

Thus, GPo1[pVK101::PP1] accumulated PHA_m and PHB chains in separate granules, in agreement with the freeze-fracture EM data.

Table III. Relative monomer composition of the polymer granules isolated from GPo1[pVK101::PP1] after sucrose density centrifugation.

	Relative monomer composition of PHA[1] (mol %)		
	3-OH-C_4	3-OH-C_6	3-OH-C_8
high density band	100	< 1.0	< 1.0
low density band	< 1.0	11.9	88.1

[1] 3-OH-C_4= 3-hydroxybutyrate; 3-OH-C_6= 3-hydroxyhexanoate; 3-OH-C_8= 3-hydroxyoctanoate.

Density of whole bacterial cells

Freeze-fracture electron microscopy generally showed either PHA_m or PHB granules in different cells of GPo1[pVK101::PP1], suggesting that cultures of GPo1[pVK101::PP1] are heterogeneous. If this is so, PHB containing cells are expected to have a relatively high density, while PHA_m containing cells should have a relatively low density. To verify this, cultures of cells with different PHA compositions were subjected to Percoll density gradient centrifugation, which is very useful in the determination of whole cell densities [20]. This method has been used to show that the buoyant cell density of *Alcaligenes eutrophus* increased when the cells accumulated PHB [21].

Fig. 7 shows the results of these density gradient centrifugation experiments. The GPp104 culture, consisting of cells which contained no polymer, equilibrated in a single sharp band, indicating that these cells were homogeneous with respect to density. The other three cultures showed broad bands, indicating that individual cells had different densities, probably due to variations in the polymer content of these cells. The GPo1[pVK101::PP1] and GPp104[pVK101::PP1] cultures showed an additional sharp band with the same density as that of the PHA^- mutant GPp104. This band is probably due to plasmid-free cells which did not form polyesters.

The mean density of GPo1 cells, which accumulated only PHA_m, was somewhat lower than the density of polymer-free cells. In contrast, the PHB accumulating cells of GPp104[pVK101::PP1] were somewhat more dense than the polymer-free cells. GPo1[pVK101::PP1] cells, which accumulate both PHB and PHA_m, have a density intermediate between that of cells containing only PHB or only PHA_m. Since the

GPo1[pVK101::PP1] culture did not show a band at the same density as the PHB accumulating cells of GPp104[pVK101::PP1], it is not very likely that individual cells of GPo1[pVK101::PP1] contain exclusively PHB as was suggested by the electron micrographs.

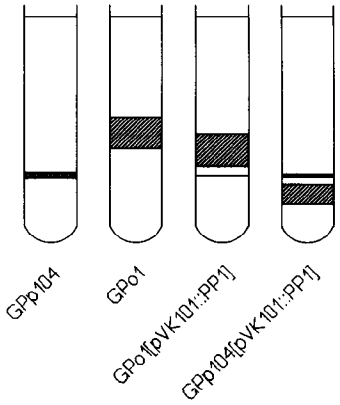


Figure 7. Percoll gradient centrifugation of cells without any polymer (GPp104) and cells with PHA_m only (GPo1), PHA_m and PHB (GPo1[pVK101::PP1]) and PHB only (GPp104 [pVK101 ::PP1]).

Therefore, PHA_m and PHB granules are not segregated between different cells of GPo1[pVK101::PP1]. Instead, individual cells contain both PHA_m and PHB granules. These are apparently present in varying mass ratios and amounts, resulting in a range of cell densities, intermediate between cells with only PHA_m or only PHB granules.

DISCUSSION

When *P. oleovorans* is equipped with the PHB biosynthetic genes, it is able to form blends of PHA_m and PHB [11]. To resolve the question whether *in vivo* PHA_m and PHB are stored together or in separate granules, we examined polyester granules by freeze-fracture electron microscopy of PHA_m, PHB and PHA_m/PHB forming strains. We also determined the density of intact granules. Finally, we measured the density of whole cells which contain different polyester granules.

Freeze-fracture electron microscopy and Percoll density gradient centrifugation

Freeze-fracture electron microcopy of a PHA^- mutant equipped with the PHB bio-synthetic genes revealed PHB granules which were deformed into the characteristic needle shapes seen earlier by Dunlop and Robards [12], whereas the stretched PHA_m granules of the wild-type

P. oleovorans appeared as mushroom-type structures, previously described by de Smet *et al.* [4].

The temperature of -100°C at which the freeze-fracturing procedure is carried out, is far below the glass transition temperature (T_g) of solvent cast PHA_m and PHB, which were found to be -37 and 2°C, respectively [10]. The fact that both PHA_m and PHB show elastic properties at -100°C suggests that the effective T_g for these polyesters *in vivo* is lower than that of the solid materials. This idea was supported by the NMR studies on *in vivo* PHB [22], but was recently contradicted by calorimetric analysis of *in vivo* PHB and PHA_m [23]. In this study, it was shown that T_g of solid and *in vivo* PHAs was identical. How the polester granules *in vivo* can be deformed at a temperature far under their T_g and why PHB and PHA_m granules show a difference in stretch deformation remains unknown.

When the recombinant strain GPo1[pVK101::PP1], which forms both PHB and PHA_m, was subjected to freeze-fracture electron microscopy, both types of deformed granules could be detected. Thus, PHA_m and PHB chains segregate in separate granules. Segregation of PHA_m and PHB has also been observed *in vitro*: differential scanning calorimetry measurements on blends of solvent cast PHA_m and PHB films showed two melting points which were identical to those of pure PHA_m and PHB. This is due to a phase separation between PHA_m and PHB chains, which indicates that PHA_m and PHB are not miscible (G.J. de Koning, unpublished results).

The separate PHA_m and PHB granules produced by GPo1[pVK101::PP1] are observed jointly in about 10 to 20 % of the cells examined by freeze-fracture electron microscopy, such single cells showing both needle and mushroom-type deformations. In most cases however, individual cells showed either PHA_m or PHB granules. Using Percoll density gradient centrifugation, cells that contained exclusively PHB could not be detected. It is therefore most likely that individual cells of GPo1[pVK101::PP1] generally contain both PHA_m and PHB granules. Although this was not evident from the electron micrographs of this strain, this is probably due to the fact that a fracture plane through a cell deforms only a few of the granules in that cell.

Sucrose density gradient centrifugation

The density of a PHA_m granule isolated from GPo1 was determined to be 1.05 mg/mL, whereas a PHB granule from GPp104[pVK101::PP1] showed a density of 1.19 mg/mL. The

latter value is similar to the density of intact PHB granules surrounded by their membrane as measured in NaBr gradients [24] and close to the density of amorphous PHB (1.177 g/mL) determined by Barham *et al.* [25]. When extracts of GPo1[pVK101::PP1] were subjected to sucrose density centrifugation, two white bands appeared: a low density band of granules consisting exclusively of PHA_m and a high density band of granules which contained only PHB. Thus, both sucrose density gradient centrifugation and freeze-fracture electron microscopy indicate that PHA_m and PHB are stored in separate granules *in vivo*.

Conclusion

In this paper we showed that a recombinant *P. oleovorans* which is equipped with the PHB biosynthetic genes is able to accumulate both PHB and PHA_m. It was established by freeze-fracture electron microscopy and sucrose density centrifugation that PHB and PHA_m are stored separately in different granules. Accordingly, the blend formation described by Timm *et al.* [11] must take place during the isolation and preparation of the polymer. Whether the *in vivo* chain segregation described in this paper is a general phenomenon which occurs during the formation of other blends, such as the poly(3-hydroxy-5-phenylvalerate)/PHA_m mixture formed by wild-type *P. oleovorans* [26], remains to be elucidated. It is clear from our studies that PHB and PHA_m show different physical properties *in vivo*, as they do *in vitro*.

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